Document name: Specification

Title of the invention: IMMUNOASSAY FOR BNP

What is claimed is:

1. An immunoassay specific for mammalian γ-BNP derivatives, characterized in that it uses, among antibodies reactive with said derivatives, the first antibody reactive with low molecular weight α-BNP having natriuretic activity which is produced by cleaving prepro-BNP or γ-BNP at the carboxyl terminus of processing signal sequence and the second antibody not reactive with α-BNP.

2. The immunoassay of claim 1, wherein the γ-BNP derivatives comprise the amino acid sequence shown by the amino acid Nos. 73-102 of SEQ ID NO: 1.

3 The immunoassay of claim 2, wherein the second antibody is specific for the amino acid sequence shown by the amino acid Nos. 73-102 of SEQ ID NO: 1.

4. The immunoassay of any one of claims 1 to 3, wherein at least one of the first and the second antibodies is detectably labeled.

5. The immunoassay of any one of claims 1 to 3, wherein the detectable label is a radioactive isotope, an enzyme, a fluorescent substance, a luminescent substance, or a particle.

6. A kit for immunoassay specific for mammalian γ-BNP derivatives, characterized in that it comprises, among antibodies reactive with said derivatives, the first antibody reactive with low molecular weight α-BNP having natriuretic activity which is produced by prepro-BNP or γ-BNP at the

carboxyl terminus of processing signal sequence and the second antibody not reactive with α-BNP.

7. The kit of claim 6, wherein at least one of the first and the second antibodies is detectably labeled and comprises a means for detecting the label.

Detailed explanation of the invention:

[0001]

Technical field to which invention pertains:

The present invention relates to an immunoassay for the brain natriuretic peptide (BNP) which is a member of natriuretic peptide family, more specifically, it relates to an immunoassay for pro-BNP (hereinafter referred to as γ-BNP) and derivatives thereof.

[0002]

Prior art and problem to be solved by the invention:

Natriuretic peptide family includes three ligands, i.e., atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and type C natriuretic peptide (CNP). Among them, ANP and BNP are cardiac hormones which are mainly biosynthesized in and secreted from the heart. ANP and BNP are similar in structure. ANP is a peptide of 28 amino acids with a ring (circular) structure formed by a disulfide bond between the 7th and the 23rd cysteine residues, while BNP is a peptide of 32 amino acids with a ring structure formed by a disulfide bond between the 10th and the 26th cysteine residues. It has been considered that at least the ring structure is required for the expression of activity. These mature peptides of 28 and 32

amino acids have been considered to be produced from respective precursor when a leader sequence is cleaved off intracellularly or at the time of secretion. That is, there has been reported that human BNP is first synthesized as a preprohormone (hereinafter, referred to as prepro-BNP) in myocardial cells, which is split before or at the time of secretion between Ser^{26}-His^{27} to give pro-BNP (hereinafter, referred to as γ-BNP), and which is further split between Arg^{102}-Ser^{103} to give BNP-32 (hereinafter, referred to as α-BNP) and BNP(1-76), and that the former exhibits the activity.

[0003]

The secretion of cardiac hormones being stimulated by various heart diseases, it well reflects the change in the cardiac functions. The secretion of ANP is accelerated mainly when the atrium undergoes a load, while the biosynthesis and secretion of BNP are stimulated when the ventricle undergoes a load. Accordingly, both ANP and BNP are useful as indicators in the diagnosis of heart disease. As the progress of the investigation of the mechanism of action in the respective hormones, the advantageous features of BNP as an indicator for diagnosing heart disease have become clear. For example, the blood concentration of BNP is only 1/6 of ANP in a normal subject but it becomes higher than ANP in patients of heart failure or the like; the blood concentration of BNP increases in the case of heart failure like ANP, and the plasma concentration of BNP often exceeds that of ANP reflecting more accurately the severity of heart dysfunction; the plasma concentration of both

ANP and BNP elevates in peripheral blood and the elevation rate is higher in BNP. Moreover, BNP level in patients of heart failure sometimes increases to several tens times to several hundreds times of that of healthy normal subjects, and the change of BNP in the cases of heart failure is so marked that no other hormones are incomparable therewith. For these reasons, the usefulness of BNP measurement has been suggested (Y. Saito et al., Mebio, 12(5), 28, 1995).

[0004]

Under the conditions, an immunoassay that utilizes BNP antibodies was provided in order to serve the assay as the diagnosis of cardiac insufficiency (Japanese Patent Publication (KOHYO) No. 507210/1995). In this method, the antibodies against the portion(s) essential for the expression of activity are not used, and thus γ-BNP or γ-BNP (1-76) produced by biodegradation by protease or the like is measured. Accordingly, as the method of measuring BNP that requires the accurate diagnosis of cardiac insufficiency or the like by determining the hormone activity, the reliability is doubtful.

In the measurement of α-BNP having natriuretic activity, the reliability of the method using a polyclonal antibody ("BNP-32", Peninsula) is doubtful as the method of measuring BNP that requires the accurate diagnosis of cardiac insufficiency or the like, because degradation products of α-BNP including fragments lacking activity due to the deletion of C-terminal region are also measured.

On the other hand, taking the above matters into

consideration, there is the measurement method of BNP using an antibody recognizing the structure essential for the expression of activity ("SHIONORIA BNP", Shionogi). However, this method would be affected significantly by the process for collecting and storing blood samples, because α-BNP is extremely instable in the collected blood. It is, therefore, necessary that the samples should be specifically treated by, for instance, adding an agent for inhibiting degradation into blood collecting tubes or maintaining the samples at low temperatures so as to obtain reliable data. Such procedures may hamper the extensive clinical application. Further, there was fear that accurate values cannot be obtained.

[0005]

Means of solving the problem:

The present inventors have conducted research intensively for the purpose of establishing an accurate method of diagnosing cardiac diseases involving BNP and found that BNP exists in blood in the form of γ-BNP or its degradation product which at least comprises structurally the α-BNP moiety (hereinafter, they are referred to as "γ-BNP derivative"), and not in the form of α-BNP which has so far been considered to be dominant. The inventors have also found that γ-BNP is more stable than α-BNP in blood, that is, one role of the N-terminal structure of γ-BNP, among many, would be the stabilization of BNP. The above indicates that an organism biosynthesizes at least 2 kinds of BNP molecule which share the BNP activity but differ in half-life. These findings led the present inventors

to have a view that it is indispensable to establish a method specific for not only α-BNP but also γ-BNP to achieve an accurate diagnosis of cardiac diseases. Thereby, the present invention was accomplished.

[0006]

That is, the present invention provides an immunoassay specific for mammalian γ-BNP derivatives, characterized in that, among antibodies reactive with said derivatives, the first antibody reactive with low molecular weight α-BNP having natriuretic activity which is produced by cleaving prepro-BNP or γ-BNP at the carboxyl terminus of processing signal sequence and the second antibody not reactive with α-BNP.

[0007]

As used herein, the term "mammalian α—BNP" refers to a peptide of low molecular weight having natriuretic activity which is derived from mammalian prepro-BNP or γ-BNP through the removal of N-terminal region as a result of processing at the carboxyl terminus of processing signal sequence. In case of human BNP, α—BNP is a peptide consisting of C-terminal 32 amino acids (Nos. 103-134) of the amino acid sequence of SEQ ID NO: 1 and having a ring structure. The carboxyl terminus of processing signal sequence on the prepro-BNP molecule varies slightly depending on the species. For example, it is No. 102 Arg in case of human BNP while it is No. 100 amino acid in case of porcine or canine BNP.

As used herein, the term "mammalian γ—BNP" refers

to a partial peptide of 32 amino acids corresponding to α-BNP at the carboxyl terminal region. In case of human γ-BNP, it comprises 108 amino acids from No. 27 His to No. 134 His of the amino acid sequence of SEQ ID NO: 1.

As used herein, the term "mammalian γ—BNP derivative" refers to a peptide fragment derived from mammalian prepro-BNP or γ-BNP through mainly the *in vivo* protease reaction, which fragment includes or is larger than α-BNP. Although the γ—BNP derivative would comprise a molecule of the same or smaller size compared to γ—BNP in general, it may comprise a fragment larger than γ-BNP. Otherwise specifically mentioned, as used herein, the term "γ-BNP derivative" includes γ-BNP itself.

[0008]

Mode for carrying out the invention:

It is preferable that a γ—BNP derivative, a target substance to be assayed, comprises a partial amino acid sequence shown by the amino acid Nos. 73-134 of SEQ ID NO: 1. The reason for this is as follows: The experiment results suggest the presence of derivatives, each obtained by the cleavage at Arg^{47}, Lys^{53} and Arg^{72} among those positions that can be cleaved by protease for γ-BNP, and the absence of a derivative generated by the cleavage at the protease cleavage position(s) existing downstream from the amino acid No. 73. That is, the results of the assay for α-BNP using the fractionation by gel filtration HPLC of samples show that the peaks of α-BNP and γ-BNP are sharply separated. Therefore, it is presumed that γ-BNP

derivatives obtained by the cleavage at the position downstream from the amino acid No. 73 do not exist or, even if they exist, the amount thereof is minute (see Figs. 1 and 3).

[0009]

The term "stable", when used herein in connection with BNP, means that a BNP molecule maintains the C-terminal ring structure including C-terminus of BNP and the natriuretic activity after undergoing the degradation by protease, and that said activity is not significantly decreased even 24 hours from the collection of blood samples. In light of this definition, the γ-BNP derivative as the target substance (analyte) of the present immunoassay is stable.

On the other hand, the term "unstable" means that a BNP sample undergoes degradation by protease at the C-terminal region and that the natriuretic activity is significantly decreases 24 hours from the collection of blood samples. In light of this definition, α-BNP is unstable.

[0010]

In one embodiment of the method of the present invention, among antibodies reactive with γ-BNP derivatives, the first antibody reactive with mammalian α-BNP and the second antibody not reactive with α-BNP are used.

Antibodies used in the present method can be monoclonal or polyclonal antibodies. The first antibody can be prepared according to a method known in the art using as an antigen human α-BNP which is commercially available or chemically synthesized, or a partial peptide thereof.

Alternatively, a monoclonal antibody appended to a commercially available α-BNP assay kit for measuring α-BNP ("SHIONORIA", Shionogi) is also available, which is reactive with the C-terminal region of α-BNP.

As the second antibody, any antibody that meets the conditions above can be used. Preferred examples of such antibody include those specific for the amino acid sequence shown by the amino acid Nos. 73-102 of SEQ ID NO: 1. The preparation of such an antibody can be carried out by any one of methods known in the art.

[0011]

The assay of the present invention can be either a competitive- or sandwich-assay and an antibody to be used may be a monoclonal- or polyclonal-antibody.

At least one of the first and the second antibodies may be labeled detectably on a solid support.

The method for labeling or immobilizing an antibody is known to one ordinary skilled in the art. Examples of the label include without limitation radioactive isotopes, enzymes, fluorescent substances, luminescent substances, and particles. The labeling of an antibody can be carried out according to a method known to one ordinary skilled in the art, for example, that described by Kono et al. (Kaku-Igaku Gijutu, 13(1), 2, (1993)).

[0012]

The present invention further provides a kit for immunoassay specific to mammalian γ-BNP derivatives,

characterized in that it comprises two antibodies wherein the first antibody reactive with mammalian α-BNP and the second antibody reactive with mammalian prepro-BNP or γ-BNP derivatives and not reactive with α-BNP.

[0013]

The kit of the present invention can be for a competitive- or sandwich-assay and the antibody to be used may be a monoclonal- or polyclonal-antibody.

At least one of the first and the second antibodies may be labeled detectably on a solid support and may further contain a means for detecting the label. Examples of the label include without limitation radioactive isotopes, enzymes, fluorescent substances, luminescent substances, or particles.

The following examples and test examples are provided to further illustrate the present invention, without limiting the scope thereof.

[0014]

Example 1

Measurement of γ-BNP Derivatives by Sandwich IRMA

Throughout the following Examples, the ordinary reagents used are of special grade supplied by Wako Pure Chemicals Industries, Ltd. or Nacalai Tesque, Inc. The bovine serum albumin (BSA) was purchased from Sigma.

(1) Preparation of Plasma Sample

1) Venous blood was collected from patients of cardiac disease or healthy volunteers and placed in blood-collecting tubes containing EDTA and aprotinin (500 KIU/l, Sigma) derived

from bovine lung. The tubes were centrifuged (x2000 g at 4°C) for 5 minutes with H-107RGA (Kokusan) to separate blood cells. Further, as to blood sampling from healthy volunteers to be used for Experiment 1, venous blood was collected from healthy volunteers and placed in blood-collecting tubes containing EDTA in the absence of aprotinin (500 KIU/l, Sigma) derived from bovine lung. The same procedures as above were repeated to obtain plasma samples. The resultant plasma samples were freezed and stored at -80°C until use.

[0015]

2) The plasma samples prepared in 1) above from the patients of cardiac disease or the healthy volunteers were fractionated by gel filtration HPLC system LC10A (Shimadzu) equipped with Superdex 75 10/30 column (Pharmacia). After equilibrating the column with 0.1 M phosphate buffer (pH 7.5, 0.3M NaCl, 5 mM EDTA) at a flow rate of 1 ml/min, 1 ml of the plasma sample was injected and 1 ml each of the effluent eluted from the column was collected. Each fraction was subjected to the measurement by assay systems for measuring γ-BNP or α-BNP as described in (2)-2) and (2)-3) below, respectively.

[0016]

(2) Assay System For Measuring γ-BNP or α-BNP

1) In the assay system, the following peptides, antibodies and kits were used.

・Human α-BNP (Peptide Institute)

・Antibody against the amino terminal region of γ-hBNP (amino acid Nos. 27-64 of SEQ ID NO: 1) (Peptide Institute)

・Monoclonal antibody against the carboxyl terminal structure of α-BNP (BC203). BC203 is an immobilized antibody appended to SIONORIA BNP kit (Shionogi), wherein a monoclonal antibody directed to the carboxyl terminal structure of α-BNP is immobilized on beads.

・Monoclonal antibody against the ring structure of α-BNP (KY-BNPII). KY-BNPII is a monoclonal antibody appended to the SIONORIA BNP kit (Shionogi), which is directed to the ring structure (112-128) of α-BNP, and is labeled with ^{125}I. This antibody is used as an immobilizing antibody.

[0017]

2) Labeling an antibody against amino terminal portion (Nos. 27-64) of γ-hBNP with ^{125}I was carried out as follows:

IgG was purified from anti-serum (Peptide Institute) raised against the amino terminal portion (amino acid Nos. 27-64 of SEQ ID NO: 1) of γ-hBNP using MASPII kit (Bio-Rad) and displaced with 0.5 M phosphate buffer (pH 7.5) using Centricon 30 (Amicon). The labeling of antibody was carried out by the chloramine T method. To a glass tube was dispensed 170 μl of purified IgG solution (77.6 μg, IgG), and10 μl of $Na^{125}I$ solution (34.2 MBq, Amersham) was added. After the addition of 0.1 % chloramine T solution (20 μl), the mixture was vigorously stirred at room temperature for 30 seconds. The reaction was quenched by adding 20 μl of 0.25 % sodium pyrosulfite solution and 20 μl of 5% aqueous potassium iodide solution. When the reaction mixture was treated with Ampure

SA column (Amersham) to remove unreacted ^{125}I and to desalt, solution containing ^{125}I-labeled antibody was obtained.

[0018]

3) Measurement of Plasma Fraction by Assay System for α-BNP

The measurement of α-BNP was carried out by commercially available "SHIONORIA BNP kit" (Shionogi). The assay is based on sandwich IRMA (Immunoradiometric Assay) which uses a monoclonal antibody KY-BNPII specific for the ring-structure of α-BNP and another monoclonal antibody BC203 specific for the carboxyl terminal structure of α-BNP. The assay was carried out in accordance with the supplier's instructions.

That is, 100 μl each of samples to be assayed or standard solutions (0, 4, 10, 150, 600 or 2000 pg/ml of α-BNP solution) were dispensed into a polystyrene test tube. To the test tube was added 200 μl of iodine-labeled anti-BNP antibody (^{125}I) solution, followed by the addition of one polystyrene bead on which anti-BC203 antibody has been immobilized. The mixture was stirred and allowed to react for 18 hours at 4°C. After washing twice with 2 ml of washing solution, radioactivity was measured on γ-counter ARC-600 (Aloka). The results are shown in Figs. 1 and 2.

[0019]

4) Measurement of Plasma Fraction by Assay System for γ-BNP

The assay was based on sandwich IRMA (Immunoradiometric Assay) which uses an antibody against the amino terminal region of γ-hBNP (amino acid Nos. 27-64 of SEQ ID NO: 1) labeled with ^{125}I and polystyrene beads on which an antibody recognizing the carboxyl terminal structure of α-BNP (BC203).

To a polystyrene tube was placed 100 μl each of samples to be assayed, 200 μl of 0.1 M phosphate buffer (pH 7.5, 0.3M, 5 mM EDTA, 0.2% BSA and 500 KIU/l bovine lung aprotinin (Sigma)) and one polystyrene bead on which BC203 antibody has been immobilized were added. The mixture was stirred and allowed to react for 18 hours at 4°C. After washing twice with 2 ml of washing solution, 300 μl of ^{125}I-labeled antibody solution was added. The mixture was stirred and allowed to react for 18 hours at 4°C. After washing twice with 2 ml of washing solution, radioactivity was measured on γ-counter ARC-600 (Aloka) The results are shown in Fig. 3.

[0020]

(3) Results

Figs. 1, 2 and 3 show the chromatograms of gel filtration HPLC of plasma samples obtained from the patients, wherein A is the position of the elution of α-BNP.

Fig. 1 shows the results of the measurement conducted by the α-BNP assay kit described in (2)-3) above. In the figure, the vertical axis represents the concentration of BNP-like substances in each fraction and the horizontal axis the volume of the effluent eluted from the column as measured

by SHINORIA BNP kit. The solid triangle, open square, and open rhombus respectively represent the measurements in different plasma samples.

Fig.2 shows the results of the measurement conducted by the α-BNP assay system described in (2)-3) above in samples different from those shown in Fig. 1. In the figure, the vertical axis represents the concentration of BNP-like substances in each fraction and the horizontal axis the volume of the effluent eluted from the column as measured by SHINORIA BNP kit. The solid triangle and solid square respectively represent the measurements in different plasma samples.

Fig.3 shows the results of the measurement conducted by γ-BNP assay system described in (2)-4) above in the same samples as shown in Fig. 2. The vertical axis represents the radioactivity measured by the γ-BNP immunoassay system and the horizontal axis the volume of the effluent eluted from the column. The solid circle represents the measurements of α-BNP.

From Figs. 1 and 2, it is revealed that there exist substances of molecular weight larger than α-BNP and having BNP-like immunoreactivity in the plasma of patients of cardiac disease, and that they are the major substances having BNP immunoreactivity.

From Fig. 3, it is revealed that the immunoassay specific for γ-BNP of the present invention can detect the major substances with BNP immunoreactivity, but cannot α-BNP at all.

The results above indicate that the immunoassay for

γ-BNP of the present invention is insensitive to α-BNP but specific to γ-BNP. Further, it has also been revealed that γ-BNP is the major substance having BNP immunoreactivity.

[0021]

Test Example 1

Stability of γ-BNP and α-BNP in Plasma

Fractions suspected to contain γ-BNP were collected from those obtained by treating plasma samples collected from patients of cardiac disease by gel filtration HPLC. The fractions were added to plasma samples obtained from healthy volunteers in the absence of bovine lung aprotinin (the minimum detection limit of α-BNP < 4 pg/ml). Then, the plasma sample was allowed to stand for 0, 2, 6, 24 hours at room temperature (25°C). The stability of BNP derivatives was evaluated by determining the BNP immunoreactivity in the plasma sample by means of SHIONORIA BNP kit.

Separately, the stability of α-BNP was evaluated using a plasma sample prepared by adding chemically synthesized α-BNP to plasma collected from healthy volunteers in the absence of bovine lung aprotinin as described above, and the plasma sample was allowed to stand for 0, 2, 6 and 24 hours at 4°C. The BNP immunoreactivity in the plasma sample was determined by SHIONORIA BNP kit in the same manner as above.

The stability of γ-BNP and α-BNP in plasma samples are shown in Figs. 4 and 5, respectively.

From Fig. 4, it is revealed that γ-BNP does not lose significantly the immunoreactivity compared with the

initial activity after 24-hour-standing at 25°C. From Fig. 5, by contrast, it is revealed that α-BNP loses the immunoreactivity to about 40% based on the initial activity after 24-hour-standing at 4°C.

The above results demonstrate that α-BNP is far less stable compared with γ-BNP in blood and that the latter is much more suited in the diagnosis of cardiac diseases than the former.

[0022]

Effect of the invention:

According to the immunoassay specific for γ-BNP of the present invention, stable and reliable clinical data are obtained without being affected by the process of collecting or storing samples, or the time from the collection until measurement. Further, the immunoassay of the present invention does not require any special pretreatments of blood samples and therefore gives clinical data conveniently, thereby contributing to the establishment of highly accurate diagnosis of cardiac diseases.

Brief explanation of the invention:

Fig. 1 is a chromatogram obtained in an α-BNP assay system wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample.

Fig.2 is a chromatogram obtained in an α-BNP assay system wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample, the same as those shown in Fig. 2.

Fig. 3 is a chromatogram obtained in an immunoassay specific for γ-BNP wherein gel filtration HPLC was conducted using Superdex 75 in a plasma sample.

Fig.4 is a graph showing the relationships between the storing time and BNP immunoreactivity of γ-BNP kept in human plasma at 25°C.

Fig.5 is a graph showing the relationships between the storing time and BNP immunoreactivity of α-BNP kept in human plasma at 4°C.